Increased Mortality of *Coptotermes formosanus* (Isoptera: Rhinotermitidae) Exposed to Eicosanoid Biosynthesis Inhibitors and *Serratia marcescens* (Eubacteriales: Enterobacteriaceae)

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ABSTRACT The biological control of termites may be facilitated if their highly evolved immune systems can be suppressed. Eicosanoids are C20 polyunsaturated acids that are of widespread biochemical importance, including their role in protecting insects from bacterial infection. In laboratory experiments, the eicosanoid biosynthesis inhibitors dexamethasone, ibuprofen, and ibuprofen sodium salt were each provided along with a red-pigmented isolate of *Serratia marcescens* Bizio, a bacterial pathogen, to the Formosan subterranean termite, *Coptotermes formosanus* Shiraki, by means of treated filter paper. The increased mortality that resulted with dexamethasone and ibuprofen supported, but alone was insufficient to prove, the hypothesis that the termites' immune systems were suppressed by these compounds, making the insects more vulnerable to infection by *S. marcescens*. This effect on mortality was noted only at 3.4×10^{10} colony-forming units per milliliter, a high treatment level. A significant amount of the infection and subsequent mortality may have resulted from direct contact with the bacterium and the remainder from its ingestion. Water-soluble ibuprofen sodium salt demonstrated a protective effect that was unexpected in light of the increased termite mortality observed with the relatively water insoluble, free acid form.

KEY WORDS *Coptotermes formosanus*, *Serratia marcescens*, immunity, eicosanoid, insect pathology, biocontrol

THE FORMOSAN SUBTERRANEAN termite, *Coptotermes formosanus* Shiraki, is an exotic pest introduced into the United States after World War II and is well established, mainly in and around port cities in the South and in California and Hawaii. In New Orleans, LA, the population of *C. formosanus* has increased yearly in the French Quarter and caused considerable structural damage to historic buildings (Henderson 1996). Nationwide, the costs for prevention, control, and repair that are attributable to subterranean termites are at least $1 billion annually (Su and Scheffrahn 1990). Control of termites by strategies employing their natural enemies such as pathogenic microorganisms (biocontrol) is very appealing from an environmental point of view (Grace 1997, Culliney and Grace 2000). Impairing the immune system of *C. formosanus* with a chemical may facilitate biocontrol through infection by pathogens that are either present in the termites' natural environment or that are introduced into the colony.

Serratia marcescens Bizio is a spore-forming, gram-negative bacterium commonly found in water, soil, and food; and it often displays a weak-to-moderate pathogenicity to insects (Steinhaus 1959, Grimont and Grimont 1978a). Outbreaks of *S. marcescens* infection frequently occur in laboratory-reared insects and are usually caused by strains that produce the red pigment prodigiosin (Yu 1979). Efforts have been made to increase stress on insects and thereby make *S. marcescens* a more effective pathogen. These largely ineffective efforts have included co-feeding with abrasives and acetic acid and other compounds, heating, maintaining high moisture levels, and co-applying with other bacterial pathogens (Steinhaus 1959, Stephens 1959). Termites are vulnerable to infection by *S. marcescens* (DeBach and McOmie 1939, Lund 1971, Khan et al. 1977), and it may be a useful pathogen to use as a model in studies involving termites and their immune systems.

Eicosanoid is a collective term for biologically active, oxygenated metabolites of C20 polyunsaturated fatty acids that are best known in medicine for humans, e.g., prostaglandins, lipoxins, and leukotrienes. However, eicosanoids have been detected in many invertebrate taxa where they play important biochemical roles (Rowley et al. 1998, Stanley and Howard 1998, Stanley 2000), including protecting insects from bacterial infection (Miller et al. 1994, 1996; Stanley 1998). Co-treatment of larvae of tobacco hornworms, *Manduca sexta* (L.), with the eicosanoid biosynthesis inhibitor (EBI) dexameth-

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asone and a red-pigmented strain of *S. marcescens* increased larval mortality caused by bacterial infection (Stanley-Samuelson et al. 1991). The ability to form nodules, an indicator of active insect defense against bacterial infection, was impaired in the larvae of *M. sexta* and the tenebrionid beetle, *Zophobas* sp., and adult crickets, *Gryllus assimilis* (F.), that were injected with EBIs and bacteria, including *S. marcescens* (Howard et al. 1998; Miller et al. 1996, 1999).

The objective of this study was to determine if treatment of *C. formosanus* with EBIs plus *S. marcescens* would kill the termites faster or in higher numbers than would *S. marcescens* alone. Unlike previous work with EBIs, the insect target in this study is a social insect equipped with behavioral mechanisms and immune responses for combating bacterial epizootics (Logan et al. 1990, Rosengaus et al. 1999) and the EBIs were administered through contact and normal feeding rather than by injection.

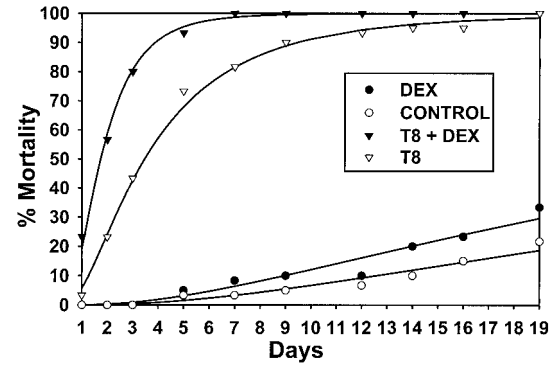

Fig. 1. Effect of *S. marcescens* isolate T8 and dexamethasone (DEX) applied by feeding/direct contact on the mortality of *C. formosanus.* Each treatment comprised 60 termites, 10 each from six different colonies.

Materials and Methods

The red-pigmented *S. marcescens* isolate T8 was obtained from cadavers in laboratory-reared *C. formosanus* termites trapped from a colony found at the Southern Regional Research Center in New Orleans, LA (Osbrink et al. 2001). The bacterium was identified (probability 100, similarity index value 0.78, distance 3.25) with the MicroLog microbial identification system (Biolog 199). Bacteria were grown on 45 nutrient agar plates at 30°C and harvested by collectively washing the plates with 120 ml of 0.1% peptone solution in water. The resulting bacterial cell suspension contained 3.4×10^{10} colony-forming units (cfu)/ml (by serial dilution) and was used undiluted. A 0.1% peptone solution was used as a diluent and as a control. The *C. formosanus* were collected from six different colonies in New Orleans, LA, using bucket traps baited with wood.

The EBIs dexamethasone, ibuprofen, and ibuprofen sodium salt were obtained from Sigma (St. Louis, MO). Ethanolic or aqueous solutions of the EBIs were prepared at a concentration of 50 μg/ml, which corresponded to 1.3×10^{-4} M dexamethasone, 2.4×10^{-4} M ibuprofen and 2.2×10^{-4} M ibuprofen sodium salt. The solutions were applied as described below.

One milliliter of dexamethasone or ibuprofen solution in absolute ethanol was applied to an 8.2-cm Whatman #1 filter paper disc (Whatman, Hillsboro, OR) in a 100 by 15-mm plastic petri plate, and it barely saturated the paper. The ethanol was allowed to evaporate, thereby depositing 50 μg of the EBI into the paper. One milliliter of either the bacterial suspension or 0.1% peptone solution was added to each plate. This treatment level corresponded to 6.4×10^8 cfu/cm^2 of *S. marcescens* on the filter paper. A total of 10 termites (nine workers, and one soldier) was then placed onto the treated paper and the plate was covered.

The water-soluble ibuprofen sodium salt was dissolved, 50 μg/ml, in the bacterial suspension. The control treatment was the ibuprofen salt, at 50 μg/ml, dissolved in a 0.1% peptone solution. Termites were placed on the paper, as described above, immediately after application of 1 ml of the ibuprofen sodium salt solutions.

Treatments consisted of bacteria alone, EBI alone, EBI plus bacteria, and peptone control. Plates were placed on wet paper towels in a closed plastic container kept at 21–23°C on a laboratory bench. The towels were rewetted as needed to maintain the relative humidity at 100%. Termites were rated as alive or dead at 2, 5, 7, 9, 12, 14, 16, and 19 d after treatment. The experiment was conducted with termites from three colonies and repeated using termites from three colonies distinct from the first three. Therefore, each treatment comprised a total of 60 termites (10 each from six different colonies).

The experimental design was a randomized complete block with eight treatments and six replicate blocks (colonies). The experimental unit to which a treatment was assigned was a petri plate containing 10 termites and treated paper. A block consisted of sets of eight petri dishes with the same *C. formosanus* source colony and different treatments. The eight treatments had a factorial structure of 4 EBIs (DEX, IBU, IBUNA, none) $\times$ 2 bacteria (T8, none).

For each of the eight treatments, probit regression analysis was used to estimate the percent mortality as a function of time. Analysis was performed using the Probit procedure of SAS (SAS Institute 1999). The probit trends based on a normal distribution and using the log transformed value for day gave good predictions of mortality. Chi-square test on regression parameter estimates for each probit model were highly significant ($P \leq 0.0001$). The P values in the test for lack-of-fit were low ($P \leq 0.05$) also indicating an adequate fit for these models.

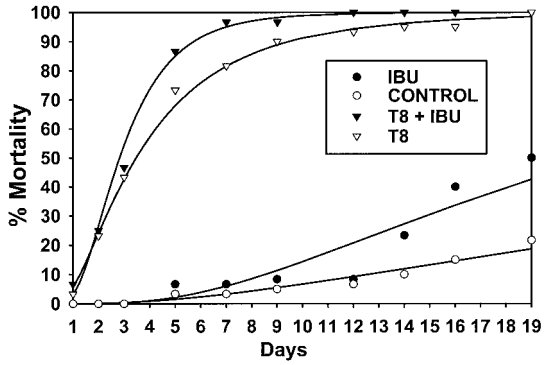

Fig. 2. Effect of *S. marcescens* isolate T8 and ibuprofen (IBU) applied by feeding/direct contact on the mortality of *C. formosanus.* Each treatment comprised 60 termites, 10 each from six different colonies.

Table 1. Predicted mortality of *C. formosanus* treated with *S. marcescens* isolate T8 and eicosanoid biosynthesis inhibitors alone and in combination

Treatment	Predicted mortality % (95% CI)		
	2 d	7 d	19 d
Control	0.12 (0.0, 1.7)	3.30 (1.4, 6.8)	18.65 (12.0, 27.3)
T8 alone	24.09 (16.2, 33.7)	81.55 (76.4, 86.0)	98.52 (96.7, 99.4)
DEX alone	0.36 (0.0, 2.3)	7.42 (4.4, 11.9)	32.47 (24.2, 41.7)
T8 + DEX	58.96 (48.7, 68.7)	98.70 (95.9, 99.7)	99.99 (99.8, 100.0)
IBU alone	0.06 (0.0, 1.6)	6.04 (2.5, 12.8)	42.53 (30.0, 55.9)
T8 + IBU	26.69 (17.8, 37.4)	95.04 (90.7, 97.6)	99.97 (99.8, 100.0)
IBUNA alone	0.00 (0.0, 0.1)	0.66 (0.1, 3.4)	27.81 (18.5, 38.9)
T8 + IBUNA	2.37 (0.8, 6.2)	23.65 (18.6, 29.3)	61.45 (53.0, 69.4)

Treatments: T8, *S. marcescens* isolate T8; DEX, dexamethasone; IBU, ibuprofen; IBUNA, ibuprofen sodium salt; control, filter paper treated with 1 ml of 0.1% peptone solution.

Results

The predicted lines for termite mortality as a function of time, along with the actual data points, areshown in Figs. 1–3. Predicted mortalities and 95% confidence intervals (CI) about the predicted values were calculated for selected time increments and are shown in Table 1. Lethal time (LT) is the value of the independent variable (day) that yields a specific mortality level. Lethal time values for 20, 50, and 80% mortality levels and 95% inverse confidence limits were calculated and are shown in Table 2 (Finney 1971). To make comparisons between the treatments, significance was declared if the confidence intervals between two treatments did not overlap.

The *S. marcescens* isolate T8 was highly virulent at the concentration used. Termite mortality was 24% by 2 d and 99% after 19 d at the conclusion of the experiment. At 50 μg/ml, dexamethasone used alone caused numerically, but not significantly, higher mortality of *C. formosanus* than the peptone control over the course of the experiment (Fig. 1; Table 1). The combination of dexamethasone and *S. marcescens* was more lethal than either treatment alone for the entire 19-d duration of the experiment, and synergistic in effect to ≈1 wk after treatment.

Termite mortality was greater with ibuprofen plus *S. marcescens* than with the bacteria alone at 7 d after treatment, and reached 100% at 19 d (Fig. 2; Table 1). Ibuprofen alone was more lethal than the control after ≈16 d and reached 43% mortality at 19 d. Ibuprofen was numerically, but not statistically, more lethal than dexamethasone.

Termites responded to treatment with ibuprofen sodium salt differently than with ibuprofen and dexamethasone (Fig. 3; Table 1). The ibuprofen sodium salt plus *S. marcescens* treatment caused much lower termite mortality than the bacterium

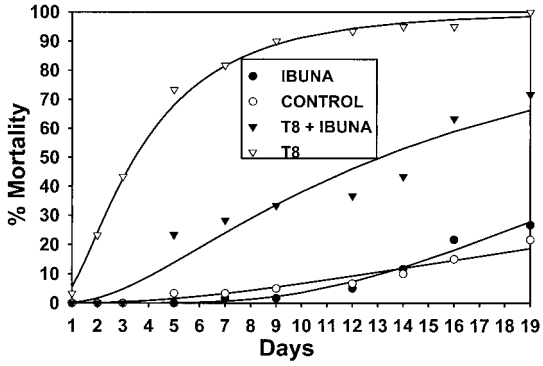

Fig. 3. Effect of *S. marcescens* isolate T8 and ibuprofen sodium salt applied by feeding/direct contact on the mortality of *C. formosanus.* Each treatment comprised 60 termites, 10 each from six different colonies.

Table 2. Predicted time to 20, 50, and 80% mortality of *C. formosanus* treated with *S. marcescens* isolate T8 and eicosanoid biosynthesis inhibitors alone and in combination

Treatment	Days[a] (95% CI)		
	LT_{20}	LT_{50}	LT_{80}
Control	20.0 (16.2, 30.8)	48.6 (31.3, 142.1)	117.9 (58.7, 676.9)
T8 alone	1.8 (1.4, 2.1)	3.5 (3.0, 3.9)	6.7 (6.0, 7.6)
DEX alone	12.9 (11.1, 15.2)	30.0 (23.1, 48.5)	70.2 (44.6, 167.6)
T8 + DEX	1.0 (0.8, 1.2)	1.7 (1.5, 2.0)	2.9 (2.6, 3.4)
IBU alone	11.8 (9.6, 14.1)	21.8 (17.5, 35.1)	40.4 (27.6, 101.4)
T8 + IBU	1.8 (1.5, 2.0)	2.8 (2.5, 3.1)	4.5 (4.0, 5.1)
IBUNA alone	16.6 (15.1, 19.1)	25.9 (21.7, 37.3)	40.5 (30.3, 75.2)
T8 + IBUNA	6.2 (5.1, 7.1)	14.2 (12.5, 16.7)	32.8 (25.9, 46.6)

Treatments: T8, *S. marcescens* isolate T8; DEX, dexamethasone; IBU, ibuprofen; IBUNA, ibuprofen sodium salt; control, filter paper treated with 1 ml of 0.1% peptone solution.

[a] Predicted times that are >19 d result from predictions outside the range of the data. Therefore, these predictions have wide confidence intervals.

alone, and only reached ≈72% at the 19-d termination of the experiment. The ibuprofen sodium salt alone resulted in the same low mortality as the peptone control.

LT_{20}, LT_{50}, and LT_{80} data are useful for comparing treatment effects on termite mortality (Table 2). Comparing the lethal time for 20% mortality includes reasonable time estimates for all treatments. Because several treatments never caused 50% or higher mortality within the observed 19 d, estimation of the time to get to greater mortalities resulted in extrapolating outside the range of observed data. This led to predictions with wide confidence intervals.

Dexamethasone plus *S. marcescens* was the most lethal treatment followed closely by ibuprofen plus *S. marcescens.* The predicted time for these treatments to kill 20% of the *C. formosanus* was 1.0 and 1.8 d, respectively. For 80% kill, the predicted time was 2.9 and 4.5 d, respectively. The *S. marcescens* used alone was next in lethality (LT_{80} = 6.7 d). Surprisingly, treatment with ibuprofen sodium salt plus *S. marcescens* was much less lethal (LT_{80} = 32.8 d) than the bacteria used alone (LT_{80} = 6.7 d). Therefore, the ibuprofen sodium salt appeared to provide some protection for the termites against bacterial infection. All the EBI treatments alone caused approximately the same mortality, which was much lower than with bacteria alone or with EBI plus bacteria treatments.

Probing work was conducted with different concentrations of *S. marcescens* and EBIs. The concentration of bacterial cells was diluted 10- and 100-fold to 4×10^9 and 4×10^8 cfu/ml, respectively, and the EBI concentration was maintained at 50 μg/ml. There was no increase in termite mortality at the10^8 cfu/ml concentration and just a slight increase at the10^9 cfu/ml concentration for the combination treatment. Increasing the EBI concentration 10-fold to 500 μg/ml while maintaining the concentration of bacteria at 10^8 cfu/ml did not result in enhanced termite mortality (data not shown).

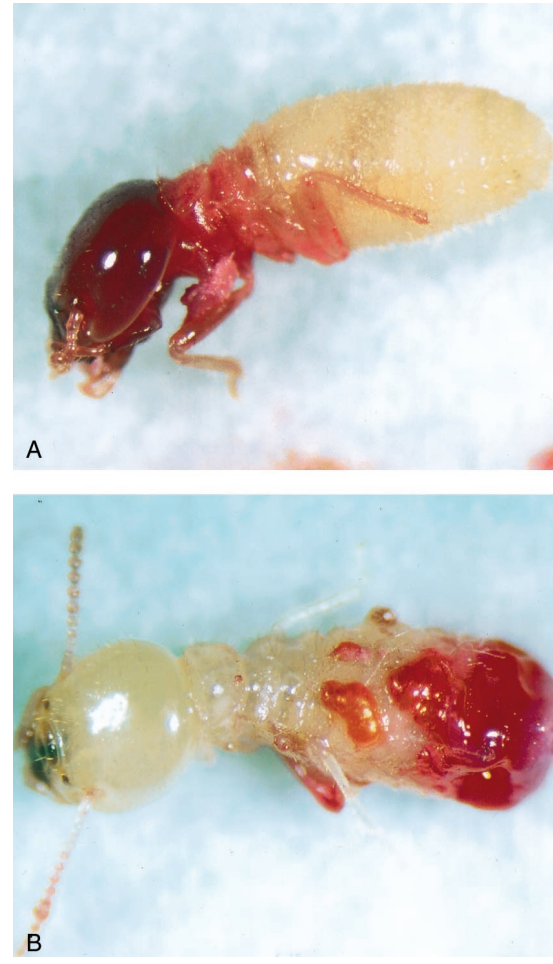

Fig. 4. (A) *C. formosanus* cadaver infested in the head, antennae, and legs with *S. marcescens.* (B) *C. formosanus* cadaver with extensive *S. marcescens* infection principally on the body.

Discussion

Coptotermes formosanus lives in intimate association with pathogenic microorganisms, including *S. marcescens* (Khan et al. 1977, Logan et al. 1990, Osbrink et al. 2001). On the one hand, biocontrol of subterranean termites by microbial pathogens may be facilitated by the warm, humid environment of the colony, their sharing of food (trophollaxis), their intimate contact with nest mates (e.g., allogrooming), and transporting infected cadavers (Grace 1994). However, termites have evolved a complex social structure, formidable immune responses, and adaptive behavior toward infected individuals so that consistently effective biocontrol by means of a single pathogen is unlikely (Logan et al. 1990). If the termite immune defense response can be suppressed, the delicate host-pathogen balance will be tipped in favor of indigenous or augmented pathogens (Ourth and Smalley 1980).

Serratia marcescens is a good model bacterium for certain types of insect biocontrol research like those involving bacterial immunity because it is weak-to-moderately pathogenic and infects a wide variety of insect species (Poinar et al. 1979, Ourth and Smalley 1980, Ourth 1988, Miller et al. 1994). The red color of cadavers and their partial liquefaction (Fig. 4) are highly suggestive of *S. marcescens* infection, which often occurs when insect vigor is reduced (Steinhaus 1959, Sikorowski and Lawrence 1998). Extracellular enzymes are important factors in its pathogenicity (Steinhaus 1959, Kaska 1976, Lysenko 1976). *S. marcescens* can occasionally overcome an insect's immune system, penetrate the gut, and cause a fatal septicemia (Flyg et al. 1980, Krieg 1987,

Ourth 1988). Used alone, this bacterium did not give encouraging results against termites in field trials (Lund 1971). Data on the mortality of insects treated with *S. marcescens* is likely to show considerable variability (Steinhaus 1959, Sikorowski and Lawrence 1998). We have noticed this in preliminary work with *C. formosanus* where mortality varied widely despite rigorous attempts to standardize treatment methodology. In this regard, care must be exercised so that the filter paper is not so wet with the bacterial suspension (somewhat viscous at 10^{10} cfu/ml) that termite mobility is impaired.

Some biotypes of *S. marcescens* are antibiotic-resistant human pathogens. These biotypes are overwhelmingly nonpigmented and are typically found in heroin addicts and in debilitated patients in hospitals (Clayton and von Graevenitz 1966, Yu 1979). Before *S. marcescens* could be used in a commercial biocontrol product, considerable research would have to be done to ensure that the biotype selected is pathogenic only to insects (Farmer et al. 1977, Grimont and Grimont 1978b, Grimont et al. 1979, O'Callaghan et al. 1996, Hejazi and Falkiner 1997).

The increased mortality of *C. formosanus* caused by dexamethasone and ibuprofen noted in the current study suggests that the insects' immune system was suppressed, making them more susceptible to infection by *S. marcescens.* These results are in agreement with previous reports that EBIs mediate insect immune system response to bacterial infection (Stanley-Samuelson et al. 1991, Miller et al. 1994, Howard et al. 1998, Miller et al. 1999). The termite infection could have originated through contact with, or ingestion of, the bacterium. Infection that was often observed as red pigmentation in antennae and legs of live and moribund termites indicated that some of the infection was caused by direct contact with the high bacterial cell concentration on the filter paper. Ingestion of high doses of *S. marcescens* causes infection in many insect species (Poinar et al. 1979, Krieg 1987, O'Callaghan et al. 1996). Because some filter paper was consumed in each of the treatment units, infection through ingestion probably contributed to the mortality we observed. However, we are unable to state the quantitative uptake of inhibitor on the paper for the different treatments.

Zootermopsis angusticollis Hagan termites fed a thick suspension of *S. marcescens* on filter paper had 50% mortality by 14 d (DeBach and McOmie 1939). The LT_{50} of 3.5 d observed in the current study for *C. formosanus* treated with *S. marcescens* T8 at 3.4×10^{10} cfu/ml on filter paper is comparable to the LT_{50} data for *Bifiditermes beesoni* Gardener, *Heterotermes indicola* Wasserman, and *Microtermes championi* Snyder, which were 5.6, 8.2, and 1.6 d, respectively, for an unspecified type of treatment with *S. marcescens* at 6×10^8 cfu/ml (Khan et al. 1977).

It may have been preferable to conduct the experiments in the dark at a higher temperature, such as 28°C, which are conditions that would be near optimum for *C. formosanus* and *S. marcescens* (Steinhaus 1959, Grace 1994). A less virulent strain of *S. marcescens* than our T8 isolate may have been a better choice to illustrate the effect that combined treatments with EBIs have on termite mortality.

Dexamethasone and ibuprofen apparently suppressed the immune system of *C. formosanus*, but the magnitude of the effect was insufficient to suggest that anything other than treatment with very concentrated bacterial suspensions would increase termite mortality. Do termites use eicosanoids as regulatory agents? We have not found any information on the presence or biochemistry of eicosanoids in termites. The evidence we have presented is indirect. This hypothesis must be tested directly by appropriate biochemical methods.

In light of higher termite mortality with ibuprofen, the apparent protective effect of its sodium salt on termite tolerance toward *S. marcescens* was unexpected. We are uncertain whether the salt's water-solubility somehow allowed it to affect the biochemistry of the termite or the virulence of the bacterium in a way that the relatively water insoluble, free acid parent compound did not. Further work in this entire area of study is encouraged and may lead to a better understanding of the biochemical mechanisms involved in the immune systems of termites.

There are numerous EBIs used in human medicine as anti-inflammatory agents. For example, dexamethasone is a synthetic adrenocortical steroid and ibuprofen is classed as a nonsteroidal anti-inflammatory agent. Combinations of other EBIs plus isolates of *S. marcescens*, or EBIs plus other bacterial or fungal termite pathogens, may be more efficacious than was demonstrated in the current study with *C. formosanus.* Combinations of EBIs and conventional insecticides should also be investigated. Because this is the first report of a pharmaceutical effect of EBIs on insects provided by contact and normal feeding rather than by injection, there are favorable implications for applied research. It may eventually be feasible to devise practical treatments involving immune system suppressants, possibly involving baits as the delivery system or formulations designed for introduction into trees and soil for control of termite colonies in locations where they cause economic losses.

Acknowledgments

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